Identification of the Quinone Cofactor in Mammalian Semicarbazide-Sensitive Amine Oxidase[†,§]

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ABSTRACT: Mammalian semicarbazide-sensitive amine oxidase (SSAO) enzymes have been classified as EC 1.4.3.6 [amine:oxygen oxidoreductase (deaminating)(copper-containing)]. However, both the identity of the quinone cofactor and the presence of copper remain unconfirmed, and SSAO has proved impossible to purify to homogeneity in sufficient yield to permit cofactor identification. To circumvent this problem, we have partially purified SSAO enzymes from bovine and porcine aortae and have established, with a redox-cycling assay, that no other quinoproteins were present in enzyme preparations. Enzymes were then derivatized with (*p*-nitrophenyl)hydrazine (*p*-NPH), which forms a covalent yellow complex with the quinone cofactor. Visible absorbance spectra of derivatized bovine and porcine enzymes (respective λ_{max} values 456 and 476 nm at neutral pH, shifting to 580 and 584 nm in 2 M KOH) were consistent with the presence of (2,4,5-trihydroxyphenyl)alanine quinone (TPQ) as cofactor. Resonance Raman spectra were essentially identical to that for pea seedling amine oxidase, a known TPQ-containing enzyme. Extensive digestion of SSAO enzymes, and of porcine kidney diamine oxidase, with pronase E yielded species with identical chromophoric properties characteristic of the dipeptide, TPQ(*p*-NPH)-Asp. Thermolytic digestion of porcine SSAO gave two cofactor-containing peptides that contained a TPQ consensus sequence, Asn-X-Asp-Tyr-Tyr, where X is a blank cycle corresponding to TPQ. N-terminal sequencing of whole enzymes revealed a membrane-spanning region typical of an extracellular type II glycoprotein. These results confirm the presence of TPQ in mammalian membrane-bound SSAO ectoenzymes.

Amine oxidase enzymes catalyze the oxidative deamination of amines, yielding an aldehyde, ammonia (or a derivative thereof), and hydrogen peroxide. Examples of this heterogeneous family of enzymes can be found throughout the animal and plant kingdoms (*1, 2*). However, their classification into subgroups, based on substrate selectivity or inhibitor sensitivity (*3, 4*), has become less meaningful as the extent to which substrates and inhibitors are shared between enzymes from different tissues and species becomes more apparent. Rather, a classification based on cofactor identity, and thus reaction mechanism, would seem to be more definitive, and at least three subgroups of amine oxidases might be defined in this way.

Perhaps the most widely studied of the amine oxidase enzymes are the mitochondrial monoamine oxidases (MAO;[1] EC 1.4.3.4), which contain a covalently bound flavin adenine dinucleotide as cofactor (*5*). Mammalian lysyl oxidase (LO; EC 1.4.3.13) contains a redox-active quinone cofactor (*6*), which has been designated lysine tyrosyl quinone (LTQ; Figure 1C), formed by posttranslational cross-linking of the ϵ-amino group of a peptidyl lysine with a side chain from a modified tyrosine residue (*7*). A third, and somewhat nebulous subgroup, has been termed the copper amine oxidases (CuAO; EC 1.4.3.6). Members of this group of soluble enzymes include diamine oxidase (*8, 9*), plasma amine oxidases (*10, 11*), and a variety of monoamine oxidase enzymes isolated from plants (*12, 13*), bacteria (*14, 15*), and yeasts (*16, 17*). Many of these enzymes have now been shown to contain the cofactor 6-hydroxydopa quinone, or TOPA quinone (TPQ) (*10*; Figure 1A), formed by a copper-

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§ Dedicated to Dr. Brian A. Callingham on the occasion of his 65th birthday.

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[1] Abbreviations: MAO, monoamine oxidase; BLAST-P, basic local alignment search tool (proteins); CuAO, copper-containing amine oxidase; DEAE, diethylaminoethyl; ϵ, molar absorption coefficient; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; LO, lysyl oxidase; LTQ, lysine tyrosyl quinone; MALDI-TOF, matrix-assisted laser desorption and ionization time-of-flight; NMR, nuclear magnetic resonance; PH, phenylhydrazine; PKDAO, porcine kidney diamine oxidase; *p*-MeOPH, (*p*-methoxyphenyl)hydrazine; *p*-NPH, (*p*-nitrophenyl)hydrazine; PSAO, pea seedling amine oxidase; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SSAO, semicarbazide-sensitive amine oxidase; TEA, triethylamine; TFA, trifluoroacetic acid; TPQ, (2,4,5-trihydroxyphenyl)alanine quinone.

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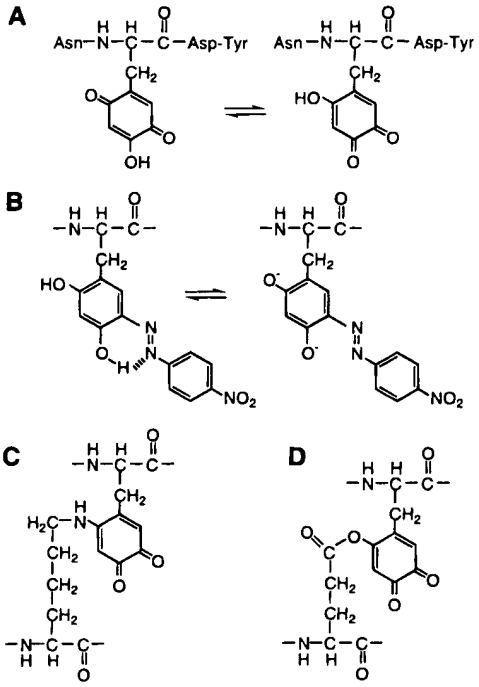

FIGURE 1: (A) Resonance structures for TPQ, with the mammalian amino acid consensus sequence. (B) The (*p*-nitrophenyl)hydrazone derivative of TPQ, showing the yellow neutral pH form (left) and the purple high pH form (right). (C) Lysine tyrosyl quinone. (D) Glutamate tyrosyl quinone.

dependent posttranslational modification of a peptidyl tyrosine residue (*18*). The use of the term "copper amine oxidases" to define members of this subgroup now requires reevaluation, since it appears that generation of LTQ is also a copper-dependent process (*7*).

While the endogenous substrates and physiological functions of most of these enzymes have been established, there remains one group of amine oxidases that has defied all attempts at classification. Semicarbazide-sensitive amine oxidase (SSAO) enzymes are associated largely with the plasma membranes of vascular and nonvascular smooth muscle cells and adipocytes (*19*−*21*). They share many biochemical and pharmacological similarities with the CuAOs in terms of substrate specificity and inhibitor sensitivity, including a susceptibility to inhibition by semicarbazide (*22*). SSAO enzymes have therefore been included as members of the EC 1.4.3.6 family, with the implication that copper is present and thus that the cofactor is TPQ. However, a visible absorbance spectral test for TPQ derivatized with phenylhydrazine (PH) or (*p*-nitrophenyl)hydrazine (*p*-NPH) (*23*) failed to confirm the presence of TPQ in bovine aorta SSAO (*24*). Furthermore, the presence of copper has not been established in these enzymes. Consequently, until now, the identity of the cofactor has remained unknown.

Further doubts were cast on the likelihood of a TPQ cofactor in tissue-bound SSAO by results from stereochemical studies of several amine oxidase enzymes. Plasma CuAOs from pig, horse, cow, and sheep catalyzed either pro-*R* or nonstereospecific proton abstraction from C-1 of tyramine, dopamine, or phenylethylamine substrates, as well as proton exchange with solvent at C-2 (*24*−*27*). Removal (wash-out) of the C-2 proton was nonstereospecific, while reprotonation (wash-in) was generally pro-*R*-specific. Several plant CuAO enzymes, as well as porcine kidney diamine oxidase (PKDAO), were pro-*S*-specific with respect to proton abstraction at C-1 but did not facilitate proton exchange at C-2 (*25, 26, 28, 29*). Bovine aorta LO, on the other hand, was also pro-*S*-specific toward tyramine but catalyzed pro-*R* wash-in/wash-out at C-2 (*30*). In contrast, when benzylamine was used as a substrate for several CuAO enzymes, proton abstraction at C-1 was pro-*S*-specific, regardless of the enzyme source (*31*). The stereochemical diversity seen within this group of related enzymes is certainly surprising and may be unique (*32*).

While rat and bovine aorta SSAOs, like the CuAOs, display pro-*S* stereospecificity toward benzylamine (*33*; G. Alton, unpublished), with tyramine as substrate, SSAO from bovine and porcine aorta catalyzed pro-*S*-specific proton abstraction from C-1 *as well as* pro-*R* wash-in/wash-out at C-2 (*34*). Thus, these aortic SSAO enzymes show a stereochemical behavior identical to that of aortic LO, which has LTQ as cofactor. The possibility that SSAO might contain LTQ, or an LTQ-like molecule, was further enhanced with the observation that TPQ can form cross-links to residues other than lysine, such as in the unusual glutamic ester of TPQ (Figure 1D) in an amine oxidase from *Aspergillus niger* (*35*).

To identify the quinone cofactors in purified CuAO and LO enzymes, previous workers have derivatized enzymes with PH or *p*-NPH, forming an intensely-colored cofactor−hydrazine covalent adduct, followed by proteolytic digestion and purification by HPLC of derivatized cofactor-containing peptides (*36*). Peptides are then examined by a combination of visible spectroscopy (*23*), resonance Raman spectroscopy (*37*), mass spectrometry (*38*), amino acid sequencing (*36*) and, if sufficient peptide is available, proton NMR (*36*). With respect to peptide sequencing, all TPQ-containing CuAO enzymes examined thus far contain the consensus sequence Asn-X-Asp/Glu (*13*), where the blank cycle, X, corresponds to TPQ, and all mammalian CuAO enzymes in which the presence of TPQ has been established contain the extended sequence Asn-X-Asp-Tyr (Figure 1A).

The ratio of SSAO to total protein present in tissues is rather low. Thus far, it has proved impossible to obtain sufficient amounts of pure enzyme from manageable quantities of raw tissue to allow characterization of active site-containing peptides, as described above. We report here the isolation and identification of the quinone cofactor in peptides derived from partially purified porcine and bovine aorta SSAO. Somewhat surprisingly, in view of the stereochemical similarities with LO, both enzymes contained TPQ, thereby inferring the presence of copper (*39*) and thus allowing their inclusion in the family of CuAO (EC 1.4.3.6) enzymes.

MATERIALS AND METHODS

Materials. Bovine and porcine aortae were obtained from Pel-Freez Biologicals, Rogers, AR, and PKDAO was from Sigma, Mississauga, ON. DEAE-Sephacel and Sepharose 4B Concanavalin-A were purchased from Pharmacia Biotech Inc., Baie d'Urfe, QC, and Sepharose 4B *Lens culinaris* lectin from Sigma. Pronase E from *Streptomyces griseus* (now classified as Protease Type XIV; EC 3.4.24.31) and thermolysin (Protease Type X from *Bacillus thermoproteolyticus*

rokko; EC 3.4.24.27) were from Sigma. An enzymatic deglycosylation kit for removal of Asn- Thr- and Ser-linked oligosaccharides (Product 80110, containing O-glycosidase DS, NANase II, and PNGase F) was purchased from Glyko, Inc., Novato, CA. All hydrazine derivatizing agents were obtained as their hydrochloride salts (>99%) from Fluka Chemical Corp., Ronkonkoma, NY. The Delta Pak C18 (5μ, 300 Å, 3.9 × 150 mm) HPLC column was purchased from Waters Chromatography, Milford, MA, and the Merck LiChroCART RP-18 (5μ, 100 Å, 4 × 125 mm) from BDH, Toronto, ON. HPLC solvents were Fisher Optima grade (Fisher Scientific, Fair Lawn, NJ) and TFA was sequanal grade (Pierce, Rockford, IL).

Partial Purification and Assay of Amine Oxidase Enzymes. Enzyme activities were determined spectrophotometrically by a peroxidase-linked assay (*40*), which is suitable for use with partially purified enzymes in the presence of detergents. Assays were made in 100 mM potassium phosphate buffer, at 37 °C and pH 7.6, with benzylamine (150 μM for assay of porcine SSAO and bovine SSAO or 2 mM for assay of pea seedling amine oxidase [PSAO]) or putrescine (1 mM for assay of PKDAO) as substrate. Absorbance changes (ϵ_{498} = 4654 M^{-1} cm^{-1}) were monitored continuously, at 498 nm in a Hewlett-Packard 8451A diode array spectrophotometer or at 490 nm in a Molecular Devices THERMOmax microplate reader. One unit of enzyme activity is defined as that catalyzing the formation of 1 μmol of substrate/min at 37 °C.

Bovine and porcine aorta SSAO enzymes were partially purified by a modification of a previously published method (*34*). Three bovine aortae (≈600 g) or 30 porcine aortae (≈900 g) were cleaned of adhering fat, ground, and washed, and the enzyme was extracted once with 1% Triton X-100 in 20 mM sodium phosphate buffer, pH 7.6, for 2 h (bovine SSAO) or 4 h (porcine SSAO), followed by centrifugation at 10000*g* for 1 h or 15000*g* for 2 h, respectively. Detergent extracts were loaded onto a 5 × 50 cm DEAE-Sephacel fast flow anion exchange column, equilibrated with 20 mM sodium phosphate buffer, pH 7.6, at 3 mL/min. The column was washed with 2 L of equilibration buffer at 2 mL/min. Bound proteins were eluted at 1 mL/min in a salt gradient, from 0 to 1.2 M NaCl in equilibration buffer over 1000 mL, followed by 700 mL of 1.2 M NaCl in equilibration buffer. Fractions of 20 mL were collected and assayed for SSAO activity, as described above. Fractions containing peak SSAO activity were pooled, and $MgCl_2$, $CaCl_2$ and $MnCl_2$ were added to a final concentration each of 0.1 mM. Pooled fractions were then applied to a 2.5 × 20 cm Sepharose 4B *L. culinaris* lectin column, as described previously (*34*), and glycoproteins were eluted with 1 M methyl-α-D-glucose in fractions of 5 mL. Fractions containing peak SSAO activity were pooled and were concentrated and washed with 20 mM sodium phosphate buffer in an Amicon ultrafiltration cell with a PM10 membrane, to a volume of less than 10 mL.

Partial purification of PKDAO was achieved by a modification of published procedures (*9, 41*). Lyophilized PKDAO (10 g) was suspended in 100 mL of HEPES (10 mM)/NaCl (70 mM) buffer, pH 7.2 (buffer A) and was dialyzed extensively (*41*). Dialysate was centrifuged at 26000*g* for 40 min, and 2 mL aliquants of the supernatant were consecutively applied to, and eluted from, a Bio-Scale Q2 anion exchange FPLC column, at 2 mL/min. Under the control of a BioLogic FPLC system, samples were washed with buffer A, and bound proteins were eluted in a salt gradient, from 100 to 400 mM NaCl in 10 mM HEPES over 10 mL, followed by 700 mM NaCl in HEPES for 6 mL. Fractions of 0.5 mL were collected additively over several runs and assayed for PKDAO activity, as described above. Fractions containing peak PKDAO activity were pooled and dialyzed extensively against 10 mM potassium phosphate buffer, pH 7, containing NaCl (150 mM) and $MgCl_2$, $CaCl_2$, and $MnCl_2$ (all at 0.1 mM) (buffer B). Pooled fractions were applied to a 2.5 × 10 cm Sepharose 4B Concanavalin-A column, equilibrated with buffer B, at a flow rate of 0.6 mL/min. Bound proteins were then eluted, in fractions of 10 mL, as follows: 100 mL of 15 mM potassium phosphate buffer, pH 7, containing 250 mM NaCl and 100 mM methyl-α-D-glucose, were applied to the column at 0.2 mL/min, followed immediately by 50 mL (one column volume) of a similar buffer containing 1 M methyl-α-D-glucose. Flow was stopped for 12 h and thereafter was resumed at 0.2 mL/min for a further 200 mL. Fractions containing peak PKDAO activity were pooled and were concentrated and washed with 20 mM potassium phosphate buffer in an Amicon ultrafiltration cell with a PM30 membrane, to a volume of less than 15 mL.

PSAO from *Pisum sativum* had been purified for a previous study and was available as referenced (*13*). Protein contents of partially purified enzyme preparations were determined by the method of Lowry et al. (*42*), with bovine serum albumin as standard.

Gel Electrophoresis of Enzymes and Assay for Quinoproteins. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of partially purified enzymes was done on freshly prepared 7.5% gels at a constant potential difference of 100 V, according to the procedure of Laemmli (*43*). Proteins were stained with Coomassie Brilliant Blue R. In some experiments, porcine SSAO was deglycosylated prior to SDS−PAGE, by incubating 108 μg of protein with 10 mU of NANase II and 2 mU of O-glycosidase DS, in 42 μL of sodium phosphate buffer (50 mM, pH 6.0), at 37 °C for 1 h. Thereafter, 5 mU of PNGase F were added to the digestion mixture and the incubation was continued for a further 24 h, at 37 °C. This procedure is designed to remove Asn-, Thr-, and Ser-linked oligosaccharides from the amine oxidase glycoprotein.[2]

Selected lanes were electroblotted to nitrocellulose membranes for quinone staining (*6*) or were electroblotted to PVDF membranes or electroeluted in 50 mM ammonium bicarbonate for N-terminal sequence analysis (*44*). Quinoproteins were detected on nitrocellulose blots by immersing membranes in 2 M potassium glycinate, pH 10, containing 0.24 mM nitroblue tetrazolium, for 30 min in the dark.

Kinetic Measurements. Michaelis constants for the metabolism of the preferred substrate, benzylamine, by bovine and porcine SSAO enzymes were determined by assaying hydrogen peroxide production over a range of substrate concentrations (*40*). Assays were done in microtiter plate wells, and contained 5 μL of enzyme in a reaction volume

[2] Sample protocols for enzymatic deglycosylation are supplied as a product bulletin; Product Specifications, Part No. 80110, Glyko, Inc., Novato, CA.

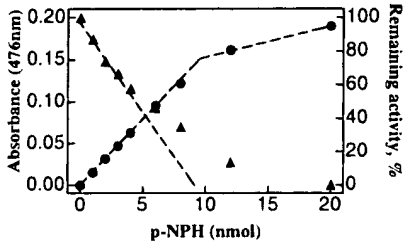

FIGURE 2: Typical titration/inhibition plot for porcine SSAO (1 mL, pH 7.6). Cofactor−(nitrophenyl)hydrazone absorbance at 476 nm (circles) and remaining activity versus benzylamine (triangles) suggest a molar absorption coefficient for this sample of 31 300 M^{-1} cm^{-1}.

of 300 μL. To confirm that these amine oxidases were susceptible to inhibition by semicarbazide, enzymes were preincubated with 100 μM semicarbazide, at 37 °C for 20 min, prior to determination of benzylamine turnover at a saturating substrate concentration. Semicarbazide was without effect on the components of the peroxidase coupling system. Results were analyzed with the linear and nonlinear regression facilities of GraphPad Prism, version 2.0 (GraphPad Software Inc, San Diego, CA).

Derivatization with (p-Nitrophenyl)hydrazine. Enzymes in 50 mM potassium phosphate buffer (pH 7.6) or 100 mM ammonium bicarbonate (pH 7−8) were derivatized by the addition of a slight excess of *p*-NPH, in several portions over a period of approximately 3 h (*23*). Derivatization was done at 37 °C in a 1 mL spectrophotometer cuvette, and the absorbance at 476 nm was read at intervals of 1 min to determine the completeness of the reaction. When derivatization was judged to be complete for each addition of inhibitor, a 5 μL aliquot of enzyme was removed and assayed for the remaining activity (*40*). When no further increase in absorbance at 476 nm was evident with continued addition of inhibitor, a 5-fold excess of *p*-NPH was added and the solution was incubated further for 15 min. Reaction mixtures were desalted on a Bio-Rad 10DG column, equilibrated with 50 mM potassium phosphate buffer or 100 mM ammonium bicarbonate and were concentrated to a volume of less than 500 μL in a 30K Microsep centrifugal microconcentrator. Since enzyme preparations contained substantial amounts of contaminating proteins, molar absorption coefficients were calculated from titration plots for SSAO−(*p*-nitrophenyl)-hydrazones (Figure 2), rather than from absorbance scans of derivatized enzymes. These varied from 31 000 to 40 000 M^{-1} cm^{-1} at 476 nm for the porcine enzyme and 34 000−40 000 M^{-1} cm^{-1} at 456 nm for the bovine enzyme. Molar absorption coefficients for the (*p*-nitrophenyl)hydrazones of PKDAO and PSAO, measured from titration plots at 464 nm, were 25 000 and 30 000 M^{-1} cm^{-1}, respectively.

Visible Absorbance Spectroscopy of Intact Proteins and Proteolytic Peptides. Visible absorbance spectra of TPQ-containing proteins and peptides, under both neutral and very basic conditions, can provide strong evidence of the presence of a TPQ cofactor (*23*; Figure 1B). Between 100 and 500 μL of derivatized enzyme, or of a lyophilized and resuspended HPLC fraction containing derivatized peptide, was added to between 200 and 500 μL of 50 mM potassium phosphate buffer, pH 7.6, and an absorbance spectrum from 350 to 650 nm was recorded. A second spectrum was recorded following the addition of a one-quarter volume of 10 M KOH to the cuvette.

Resonance Raman Spectroscopy. Resonance Raman spectra for *p*-NPH derivatives of PSAO, a known TPQ-containing CuAO, and of bovine and porcine SSAO enzymes were obtained with 150 μL sample solutions of absorbance 0.3−0.7 AU/cm at 468 nm (PSAO) or 456 nm (SSAO), filtered through a Millex-GV 0.22 μm filter, in 50 mM ammonium bicarbonate. Spectra were obtained with the first half of a Spex 1401 spectrograph coupled to a liquid-N_2-cooled CCD detector (Princeton Instruments, Trenton, NJ). Laser excitation at 457.9 nm was generated by an argon ion laser (Coherent, Santa Clara, CA), focused onto a spinning NMR tube (5 mm o.d.) containing the sample, in a 135° backscattering geometry. The laser power was typically 30 mW, spectral resolution was 5−7 cm^{-1}, and data accumulation was for a period of 400−450 s. Peak frequencies were calibrated relative to solvent standards (benzene, chloroform, and carbon tetrachloride) and are accurate to ±2 cm^{-1}. A background spectrum for 50 mM ammonium bicarbonate was subtracted from all spectra.

Proteolysis with Pronase E. Partially purified PKDAO and porcine and bovine SSAO enzymes were diluted in 50 mM sodium phosphate buffer, pH 7.6, containing NaCl (50 mM), to a total protein concentration of between 1 and 4 mg/mL. Pronase E was added at a ratio of 1:1 (w/w) with total protein, and samples were incubated at 37 °C with gentle shaking for 24 h (PKDAO) or 48 h (SSAO) (*9*). Following digestion, samples were acidified to pH 2 with HCl and were centrifuged briefly to remove particulate matter. Digests were applied to a C18 Sep-Pak cartridge and were eluted with 90% MeOH, as a bright yellow liquid. The organic phase was removed in a Savant Speed Vac prior to purification by HPLC.

Proteolysis with Thermolysin. Partially purified porcine SSAO (15 mg of total protein in 400 μL of 100 mM ammonium bicarbonate) was added to an equal volume of 10 M urea, and the mixture was incubated at 37 °C with gentle shaking for 30 min. To the denatured proteins were then added 1.2 mL of ammonium bicarbonate and 15 μL of thermolysin at a concentration of 25 mg/mL in 0.15 M $CaCl_2$ (*45*). The digestion mixture was incubated at 37 °C with gentle shaking for 65 h, with further 15 μL additions of thermolysin at 18 and 40 h. Following digestion, the yellow sample was chilled and centrifuged briefly to remove particulate matter, prior to purification by HPLC.

Isolation of Cofactor-Containing Peptides by HPLC. Purification of peptides obtained from pronase digestions was done on a Delta Pak C18 column equilibrated with 0.2 mM ammonium bicarbonate. Peptides were eluted with a gradient from 0 to 49% methanol in ammonium bicarbonate over a period of 21 min, at 0.8 mL/min. Elution of peptides was monitored at 280 nm (aromatic residue absorbance) and 465 nm (cofactor−(nitrophenyl)hydrazone absorbance at neutral pH), and fractions were collected into siliconized tubes at 1 min intervals. Methanol was evaporated from the fractions containing the majority of the cofactor adduct, and these samples were reinjected onto the same column, equilibrated with 0.2% (v/v) TFA. Peptides were eluted with a gradient from 0 to 54% acetonitrile in 0.2% TFA over a period of 22 min, at 1 mL/min. Elution of peptides was monitored at 280 and 400 nm (cofactor−(nitrophenyl)hydrazone absor-

bance at acidic pH). Fractions (1 mL) containing the cofactor adduct were dried in a Savant Speed Vac and stored under argon at −20 °C. In some cases, samples were removed from fractions prior to drying and visible absorbance spectra were obtained.

Purification of thermolytic peptides from porcine SSAO was done on a LiChroCART C18 column equilibrated with 0.3% (v/v) TEA acetate, pH 7. Peptides were eluted with a gradient from 12 to 30% acetonitrile in TEA acetate over a period of 45 min, at 1 mL/min. Elution of peptides was monitored at 280 and 476 nm. After removal of acetonitrile, fractions (1 mL) containing the majority of the cofactor adduct were reinjected onto the same column equilibrated with 0.2 mM ammonium bicarbonate. Peptides were eluted with a gradient from 10 to 25% methanol in ammonium bicarbonate over a period of 30 min, at 1 mL/min. A portion of fractions containing the cofactor adduct was removed for analysis by visible absorbance spectroscopy, and the remainder was evaporated almost to dryness in a Savant Speed Vac and was resuspended in 0.2% TFA for amino acid sequence analysis.

Amino Acid Sequence Analysis. Amino acid sequences were obtained for cofactor-containing thermolytic peptides from porcine SSAO and for whole bovine and porcine SSAO enzymes electroeluted into buffer or electroblotted onto PVDF membranes from SDS−polyacrylamide gels. Analysis by Edman degradation was done on an Applied Biosystems 476A gas-phase sequencer with on-line RP-HPLC and a 610A data analysis system. Prior to sequencing, liquid samples were adsorbed to the PVDF support of a Prosorb sample preparation cartridge and treated with 150 μg of methanolic Biobrene$^+$. Electroblotted bands containing the sample of interest were excised from membranes and treated with Biobrene$^+$.

RESULTS

Partial Purification of Amine Oxidase Enzymes. Partial purification of bovine and porcine SSAO enzymes resulted in enzyme preparations of yields (%) similar to those obtained previously (*34*), but with more enzyme activity overall as a result of the larger masses of starting materials used (Tables 1 and 2). Negligible SSAO activity remained in tissue pellets following detergent extraction and centrifugation. Anion exchange chromatography was effective in removing a substantial amount of a red-brown heme protein, as evidenced by an absorbance peak (Soret band) at 412 nm in the column washthrough. Incomplete removal of this contaminant would result in partial masking of the cofactor-hydrazine adduct absorbance in subsequent spectroscopic examinations of the enzyme. A portion (≈15%) of SSAO activity was not retained on the DEAE column and could be measured in the column washthrough. This has been seen previously in anion exchange purification of SSAO (*34*). Washthrough of enzyme from the lentil lectin column was less than 10%, suggesting that virtually all of the solubilized SSAO is glycosylated.

SDS−PAGE of SSAO preparations showed a diffuse band at about 100 kDa, as well as several other major and numerous minor bands. A test for the presence of quinoproteins on nitrocellulose electroblots from these gels showed blue-purple staining of the diffuse 100 kDa band, and in some gels, faint staining at approximately 200 kDa, corresponding to enzyme dimer. *No other quinoproteins were present in these preparations.* Densitometric measurement of SDS−PAGE gels provided estimates of the purity of the enzymes, which were 16% (porcine SSAO) and 11% (bovine SSAO).

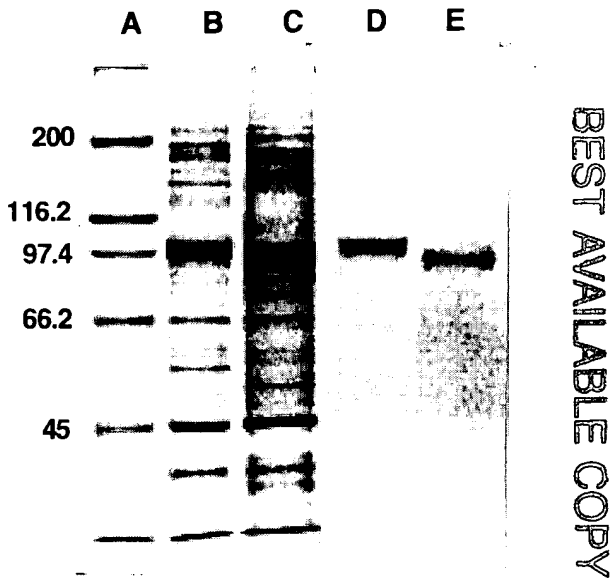

FIGURE 3: SDS−PAGE of porcine SSAO. Lane A: Molecular weight standards (values in kDa). Lane B: Porcine aorta SSAO. Two major bands, at 102.0 and 95.3 kDa, and a minor band at 92 kDa, stained positively on nitrocellulose for the presence of a redox-active quinone (lane D). Lane C: Porcine aorta SSAO following deglycosylation. A single diffuse band at 91.6 kDa stained positively for the presence of a redox-active quinone (lane E). In lanes D and E, faint staining is seen at around 200 kDa, corresponding to enzyme dimer. The intense band that appears in lane C at 80 kDa following deglycosylation may correspond to a cell adhesion (cadherin) protein.

When small amounts of porcine SSAO were examined by SDS−PAGE, the diffuse 100 kDa band could be separated into two major, well-defined bands, at 102.0 kDa and 95.3 kDa, as well as at least one very minor band at 92 kDa (Figure 3). This has been seen by others in *pure* preparations of porcine SSAO (*46*). It was confirmed that all three bands were redox-active quinoproteins. When samples were deglycosylated prior to SDS−PAGE, the multiple bands disappeared and only a single (although more diffuse) redox-active band at 91.6 kDa was visible. This suggests that the multiple quinoprotein bands are SSAO subunits in different, but distinct, glycosylation states, as opposed to contaminating proteins that might react with *p*-NPH. The lower edge of the deglycosylated band was at 86.8 kDa, suggesting that the peptide backbone of each subunit has a mass no greater than 86.8 kDa and that at least 10% of the enzyme mass can be attributed to surface carbohydrates.

To confirm that the isolated quinoprotein was indeed an SSAO enzyme, kinetic and inhibition studies were done. Michaelis constants ± standard error for turnover of benzylamine by porcine and bovine SSAO enzymes were 12.7 ± 1.5 and 7.3 ± 0.9 μM, respectively. Both enzymes were inhibited by more than 96% following preincubation with semicarbazide (100 μM).

Table 3 indicates the degree to which commercially available PKDAO was partially purified by anion exchange and affinity chromatography. SDS−PAGE revealed a major

Table 1: Partial Purification of Porcine Semicarbazide-Sensitive Amine Oxidase[a]

purification step	units	specific activity (units/mg protein)	yield (%)
Triton X-100 extraction	8.4	0.0019	100
DEAE-Sephacel fast flow	2.28	0.0032	27
Lens culinaris lectin	0.96	0.020	11[b]

[a] 900 g of starting material. [b] Estimated purity by densitometry, 16%.

Table 2: Partial Purification of Bovine Semicarbazide-Sensitive Amine Oxidase[a]

purification step	units	specific activity (units/mg protein)	yield (%)
Triton X-100 extraction	20.9	0.0028	100
DEAE-Sephacel fast flow	6.21	0.0037	30
Lens culinaris lectin	3.7	0.059	18[b]

[a] 600 g of starting material. [b] Estimated purity by densitometry, 11%.

Table 3: Partial Purification of Porcine Kidney Diamine Oxidase[a]

purification step	units	specific activity (units/mg protein)	yield (%)
dialysis	47.3	0.010	100
Q2 anion exchange FPLC	20.7	0.014	43.8
concanavalin-A	8.2	0.13	17.3[b]

[a] 10 g of starting material (lyophilized powder; Sigma). [b] Estimated purity by densitometry, 14%.

band at 97 kDa (not shown), which represented approximately 14% of the total protein present, as measured by densitometry.

Resonance Raman Spectra for Intact Enzyme−(Nitrophenyl)hydrazones. Resonance Raman spectra for *p*-NPH derivatives of PSAO and for bovine and porcine SSAO are shown in Figure 4. The most striking feature of these spectra is that they are virtually identical, suggesting that the chromophore, and thus the cofactor, are similar in these enzymes. Furthermore, allowing for small frequency shifts as a result of different active site environments, these spectra are superimposable upon those for *p*-NPH derivatives of the TPQ-containing enzymes, histamine oxidase (*47*), and phenylethylamine oxidase (*18*) from *Arthrobacter globiformis*, bovine serum amine oxidase (*48*), porcine serum amine oxidase (*13*), and PKDAO (*13*). The vibrational mode at about 1400 cm^{-1} contains some contribution from stretches localized on the C-2=O moiety of TPQ (*49, 50*). This signal was not seen in a PH-labeled thermolytic peptide from bovine aorta LO (*7*), which is linked to the N-terminal of lysine by a C−N bond at C-2 of TPQ (Figure 1C). However, signals at 1401 and 1397 cm^{-1} have been reported respectively in a trypsin/pepsin digest of bovine aorta LO−phenylhydrazone (*51*), and in the *p*-NPH derivative of an *A. niger* amine oxidase containing an ester linkage from C-2 of TPQ to glutamatic acid (*35*; Figure 1D). The identity of the bonds responsible for these signals is unclear. In the latter study, the most striking difference between the spectra for the (*p*-nitrophenyl)hydrazones of whole enzyme and synthetic TPQ was the lack of a signal at 1254 cm^{-1} in the enzyme, which has also been attributed to a mode with some C-2=O stretch contribution (*50*). Spectra obtained from LO are also without this signal (*7, 51*), and its absence can be attributed tentatively to the TPQ-peptide link at C-2 (*35*). A signal of

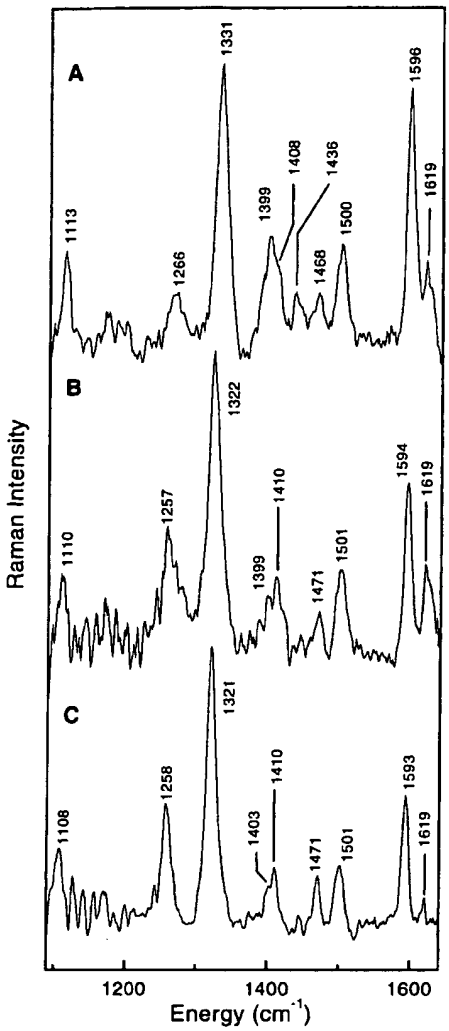

FIGURE 4: Resonance Raman spectra of (*p*-nitrophenyl)hydrazone derivatives of PSAO (A), bovine SSAO (B), and porcine SSAO (C). Spectra were obtained from approximately 20 μM enzyme and were excited with 30 mW of laser light at 457.9 nm. Total accumulation time was 400−450 s.

similar frequency is present in spectra from most other TPQ enzymes and from bovine and porcine SSAO. The mode at about 1310 cm^{-1} is also thought to contain some C-2=O stretch character (*50*) and is evident as a shoulder on the 1321−1331 cm^{-1} peaks in the SSAO spectra (Figure 4). The main signal arising from the C-2=O stretch occurs at 1575−1587 cm^{-1} (*50*), although, in *p*-NPH-derivatized enzymes, this is masked by the strong signal arising from the N=N stretch of the (*p*-nitrophenyl)hydrazone at 1590−1600 cm^{-1} (*35*). Taken together, these data suggest that the cofactor in bovine and porcine SSAO is TPQ and that modification of the cofactor resulting in a linkage from C-2 of TPQ to the peptide backbone is unlikely.

Isolation of Cofactor-Containing Peptides by HPLC. Figure 5 shows the HPLC elution profiles for pronase digests of bovine SSAO (A), porcine SSAO (B), and PKDAO (C). Left-hand panels show elution profiles obtained at 280 nm (peptide absorbance; dotted lines) and 450−474 nm (cofactor−hydrazone absorbance; solid lines), at neutral pH. Right-hand panels show profiles obtained at 280 nm (dotted lines) and 400 nm (solid lines) when the major peaks at 17.60 min

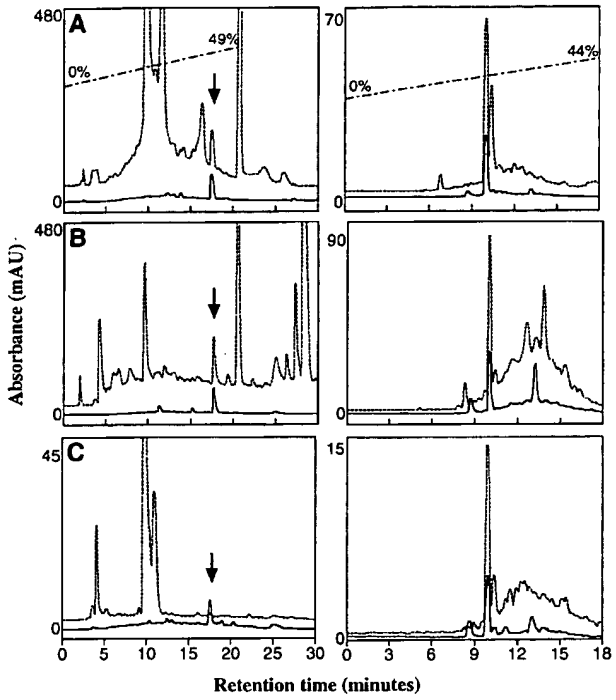

FIGURE 5: HPLC elution profiles for pronase E digests of bovine SSAO (A), porcine SSAO (B), and PKDAO (C). Left-hand panels indicate absorbances for peptides (280 nm, dotted lines) and cofactor−hydrazone adducts (450−474 nm, solid lines) eluted from a Delta Pak C18 column with an ammonium bicarbonate/methanol gradient (pH 7). Reinjection of the major cofactor-containing peptides at 17.60 min (arrowed), on the same column and with a TFA/acetonitrile gradient (pH ≈ 2), gave the elution profiles shown in the right-hand panels, with absorbances monitored at 280 nm (dotted lines) and 400 nm (solid lines). From each enzyme, a single major peak was collected at 10.05 min. Dashed lines and associated values indicate the duration and organic solvent contents of the gradients. Profiles shown on the right do not correspond to injection of all of the material collected from the first column.

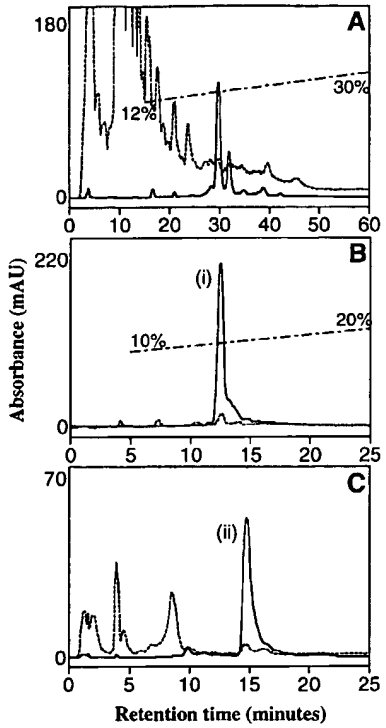

FIGURE 6: HPLC elution profiles for a thermolytic digest of porcine SSAO. The filtered digest was eluted from a LiChroCART RP18 column with a TEA acetate/acetonitrile gradient (pH 7; A). Dotted and solid lines indicate absorbance at 280 and 476 nm, respectively. Peaks containing the majority of the cofactor-containing peptides were reinjected on the same column with an ammonium bicarbonate/methanol gradient (pH 7). Reinjection of the 29.9 min peak yielded a major peptide with a retention time of 12.6 min (B), designated peptide (i), while reinjection of the 32.0 min peak showed several peptides, but only one cofactor-containing peptide with a retention time of 14.8 min (C), designated peptide (ii). Dashed lines and associated values indicate the duration and organic solvent contents of the gradients.

(arrowed) were rechromatographed at low pH. Yields obtained in 17.60 min peaks from bovine and porcine SSAO and PKDAO were 2 nmol (9%), 1.2 nmol (7%), and 0.6 nmol (10%), respectively. Under similar digestion and HPLC conditions to those used here, PKDAO yielded a major peak that was identified by electrospray mass spectrometry as the (*p*-nitrophenyl)hydrazone of TPQ-Asp (*9*). In the present study, both bovine and porcine SSAO enzymes yielded species with retention times identical to those of the PKDAO cofactor adduct, under both sets of elution conditions. Thus, digestion to completion with pronase of PKDAO and of bovine and porcine SSAO yields cofactor-containing peptides that have identical chromatographic properties and that therefore may be identical in structure. Visible absorbance spectra obtained for the porcine peptide were consistent with the presence of TPQ (see Figure 7, below). Taken together, these data suggest that the most likely identity of the species purified from PKDAO, and thus from bovine and porcine SSAO, is the (*p*-nitrophenyl)-hydrazone of TPQ-Asp.

While previous workers reported a parent ion mass of 462 ($M + H^+$) for TPQ(*p*-NPH)-Asp, as well as a fragmentation spectrum (*9*), we were unable to obtain a parent ion mass by several mass spectrometric techniques (see Discussion). On several occasions, a *m/z* signal of low intensity was seen at 462 when HPLC fractions from pronase-treated bovine SSAO were subjected to matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry (not shown). However, neither an exact mass nor a fragmentation spectrum could be obtained for this species, thereby precluding its identification.

Figure 6 shows the HPLC elution profiles for a thermolytic digest of porcine SSAO. Elution profiles obtained at 280 and 476 nm are shown for separation of the digest with a TEA acetate/acetonitrile gradient (A) and for further purification with an ammonium bicarbonate/methanol gradient of the major peaks at 29.9 min (B) and 32.0 min (C). Peaks from the latter gradient were designated **(i)** (12.6 min peak from middle panel) and **(ii)** (14.8 min peak from lower panel) and were subjected to visible absorbance spectroscopy and/or N-terminal amino acid sequencing.

Visible Absorbance Spectroscopy of Intact Proteins and Proteolytic Peptides. Visible absorbance spectra, obtained at neutral pH and in 2 M KOH, for SSAO−(nitrophenyl)-hydrazones and peptides derived thereof, are shown in Figure 7. In each case, the peak shift observed when samples are subjected to conditions of high pH is indicative of the presence of a TPQ cofactor (*23, 24*). While the pK_a for ionization of the azo group of the derivatized cofactor has been measured as 12.2 in a model TPQ hydantoin (*52*), this

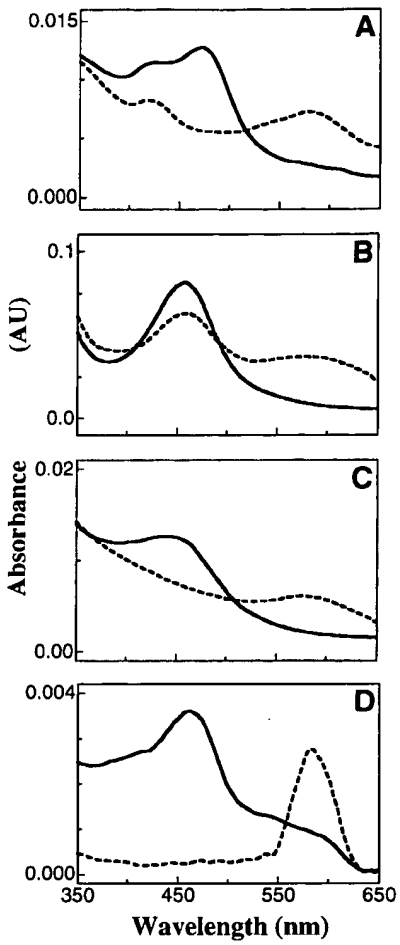

FIGURE 7: Visible absorbance spectra, obtained at neutral pH (solid lines) and in 2 M KOH (dashed lines), for (*p*-nitrophenyl)-hydrazones of porcine SSAO (A), bovine SSAO (B), a pronase-derived peptide from porcine SSAO (C; see Figure 5B), and thermolytic peptide (i) from porcine SSAO (D; see Figure 6B). Respective peak shifts were 476−584 nm (A), 456−580 nm (B), 450−580 nm (C), and 462−583 nm (D). The λ_{max} for TPQ(*p*-NPH)-Asp at neutral pH has been determined previously to be 455 nm (*9*). The effects of a contaminating heme protein can be seen clearly in (A), as an absorbance at about 420 nm.

value may be higher when the cofactor lies within the bovine SSAO active site, resulting in an incomplete shift in 2 M KOH. The visible absorbance spectrum of PKDAO−(*p*-nitrophenyl)hydrazone showed a complete red-shift of 120 nm, from 464 to 584 nm, in 2 M KOH (not shown).

Spectra have also been obtained for SSAO enzymes derivatized with PH. While addition of KOH to bovine SSAO (λ_{max} 428 nm at neutral pH) caused bleaching of the spectrum with no peak shift (*24*), an incomplete shift was seen with porcine SSAO, from 444 to 494 nm in 2 M KOH, and with no bleaching (A. Holt, unpublished results). In 4 M KOH, the peak shift to 494 nm was almost complete, suggesting that the environment in which the TPQ cofactor lies affects the pK_a of the reaction, and perhaps also the λ_{max} values at neutral and high pH. The λ_{max} values for the phenylhydrazones of LO and of an LTQ model compound at neutral pH were 454 and 452 nm, respectively (*7*), higher than values typical of TPQ-containing enzymes.

Amino Acid Sequence Analysis. The sequence of thermolytic peptide **(i)** from porcine SSAO was Asn-X-Asp-Tyr-Tyr, where X, a blank cycle, corresponds to an unidentifiable PTH derivative. Several amino acids were always seen in cycle 1 from three different preparations, although none were consistently present in equimolar amounts with Asn. Any unidentifiable peaks present in cycle 1 were very much smaller than that for Asn. The signal intensity dropped significantly after cycle 5, with the result that no amino acid could be assigned to position 6. The deduced sequence of thermolytic peptide **(ii)** was identical to that of peptide **(i)**, while all of the contaminating amino acids in cycle 1, except Val, were different from those identified in peptide **(i)**. The lack of a second residue, consistently equimolar with Asn, in cycle 1 suggests that there exists no stable linkage between TPQ and another amino acid residue in the protein. Approximately 16 pmol of Asn were visualized in cycle 1 from peptide **(i)**, with around 1.5 nmol of the peptide, purified from 20 nmol of derivatized enzyme-bound TPQ, submitted for sequencing. Low recovery rates, a result of losses during immobilization prior to sequencing, are not uncommon with such small peptides (S. Perry-Riehm, personal communication).

Protein sequence database searches were made for the pentapeptide Asn-Tyr-Asp-Tyr-Tyr, with the premise that TPQ is derived from Tyr. BLAST-P (*53*) and Blitz (*54*) searches of several nonredundant protein databases revealed no matches with known protein sequences. Thus, while the sequence, Asn-X-Asp-Tyr seems to be common to all mammalian TPQ-containing CuAO enzymes, the presence of Tyr at position 5 makes these enzymes unique within the CuAO family. Of physiological interest is the presence of Val, rather than Tyr, at the corresponding position in porcine plasma amine oxidase (*13*), indicating that in pig, at least, plasma amine oxidase is not derived from the blood vessel wall enzyme (*55*−*57*).

The N-terminal sequences obtained from bovine SSAO, and from both subunits of the porcine SSAO, are shown in Table 4. All three sequences were identical in the first 20 residues and indicate an initial hydrophilic region (residues 1−6) followed by a hydrophobic chain of at least 14 amino acids. The identical sequences obtained for the proposed SSAO subunits suggest that the subunits likely differ only in their degrees of glycosylation. A BLAST-P search revealed 95% homology in residues 1−20 of the purified SSAO enzymes with a cloned amine oxidase in adipocyte plasma membranes from *Rattus norvegicus* (*58*), previously identified as a 200 kDa membrane glycoprotein (*59*), and 90% homology with a cloned human placenta amine oxidase (*60*). Residues 7−26 are reported to correspond to a single membrane-spanning hydrophobic anchor region (*58*), and this region was highly conserved (>95%) between porcine aorta SSAO and rabbit adipocyte SSAO. The human placenta enzyme displays 83% homology toward the rat adipocyte SSAO and codes for the amino acids Asn-Tyr-Asp-Tyr-Val, a TPQ consensus sequence, from positions 470−474. Only the first 322 residues were predicted for the rat SSAO, although it seems likely that this enzyme also contains a TPQ consensus sequence. A lower degree of homology (60%) is evident between residues 1 and 20 of the SSAO enzymes and a cloned human retina-specific amine oxidase (*61*), although the degree of homology is higher within the hydrophobic anchor region. The retinal enzyme contains a

Table 4: N-Terminal Sequences of Purified SSAO and Cloned CuAO Enzymes

	bovine SSAO[a]	porcine SSAO (102)[b]	porcine SSAO (95.3)[c]	rabbit SSAO[d]	human placenta AO[e]	human retina AO[f]
1	M/S	M/S	M	M	M	M
	N/I	N/I	N	T	N	H
	Q/V	Q/V	Q	Q	Q	L
	K/V	K/V	K	K	K	K
	T/S	T/S/A	T	T	T	I
	T	T/P/E/L	T	T	I	V
	L	L	L	L	L	L
	V	V	V	V	V	A
	L	L	L	L	L	F
	L	L	L	L	L	L
11	A	A	A	A	I	A
	L	L	L	L	L	L
	A	A	A	A	A	S
	V	V	V	V	V	L
	I	I	I	I	I	I
	T	T	T	T	T	T
	I	I/V	I	I	I	I
	F	F	F	F	F	F
	A	A/T	A	A	A	A
	L	L	L	L	L	L
21		V	V	V	V	A
			?[g]	C	C	Y
			V	V	V	V
			L	L	L	L
			L/G	L	L	L
			A	A	V	T
			G	G	G	S
			R	R	R	P
			G	S	G	G
			N	G	G	G
31			D	D	D	S

[a] This study. Single (100 kDa) band from SDS−PAGE. [b] This study. Upper (102 kDa) band from SDS−PAGE. [c] This study. Lower (95.3 kDa) band from SDS−PAGE. [d] Rabbit adipocyte SSAO (cloned) (*58*). [e] Human placenta SSAO (cloned) (*60*). [f] Human retina-specific amine oxidase (cloned) (*61*). [g] ? represents an unidentified amino acid.

TPQ consensus sequence, Asn-Tyr-Asp-Tyr-Ile, from positions 464 to 468 (*61*).

The Ser-Ile-Val-Val-Ser-Pro secondary sequence, present in yields similar to those of the proposed SSAO sequences in residues 1−5 of bovine SSAO and 1−6 of the higher mass porcine subunit (Table 4), may correspond to the N-terminal of a cadherin (cell adhesion) protein. A human cell-surface cadherin of M_r 78.3 kDa has been sequenced, which, in its glycosylated state, migrates as a band at around 110 kDa in SDS−PAGE (*62*). It is thus possible that bovine and porcine aortic cadherins might comigrate with SSAO. Interestingly, the tissue distribution of human cadhedrin (*62*) is very similar to that of SSAO in human (*63*) and rat (*64*) tissues.

DISCUSSION

This study has demonstrated that a two-column partial purification of mammalian aortic SSAO permits isolation of a single redox-active quinoprotein in sufficiently high yield to enable subsequent identification of the enzyme cofactor by established methods. SDS−PAGE of pure porcine aorta SSAO (*46*) revealed at least two redox-active bands, and results presented here suggest that these are SSAO subunits in different glycosylation states. Of crucial importance is the observation that no other quinoproteins were present in these impure enzyme preparations, which might form a chromophore with *p*-NPH. Amino acid sequences for two closely related enzymes, bovine plasma amine oxidase (*8*) and human placenta SSAO (*60*), provide respective, unglycosylated subunit M_r values of 82.8 and 82.5 kDa, with 81% amino acid sequence homology (*60*). Glycosylated subunit M_r values determined here by SDS−PAGE for bovine and porcine SSAO are some 15−20 kDa higher than this, consistent with results for a rat adipocyte SSAO (*65*), which demonstrates 83% amino acid homology with the human placental SSAO (*58*). The deglycosylation procedure used here may not have removed all surface carbohydrates and it is thus not possible to compare M_r values for "deglycosylated" aortic SSAO with amino acid sequence masses from related enzymes.

Visible absorbance spectroscopy of SSAO−(*p*-nitrophenyl)hydrazones and their cofactor-containing peptides indicates a red-shift of approximately 120 nm in 2 M KOH, a consequence of ionization of the azo group of the derivatized cofactor (*23*). The *p*-NPH derivative of the C-2-linked glutamyl TPQ found in an amine oxidase from *A. niger* also undergoes a red-shift in base (*35*). However, the initial λ_{max} value of 440 nm, which is lower than would be expected for a TPQ−(*p*-nitrophenyl)hydrazone, shifts to 585 nm at high pH, a difference of 145 nm. Similarly, the red-shifts seen with *p*-NPH derivatives of other carbonyl cofactors, such as pyridoxal (422 to 541 nm) and pyrroloquinoline quinone (443 to 456 nm), are very different from those seen with TPQ enzymes, which usually have λ_{max} values ranging from 457 to 472 nm at neutral pH, and from 578 to 587 nm in KOH (*23*). Thus, absorbance spectra for SSAO enzymes are consistent with the presence of TPQ and do not support the existence of any linkage through C-2 to the peptide chain.

The incomplete red-shift seen previously with bovine SSAO−(*p*-nitrophenyl)hydrazone in 2 M KOH (*24*) is not necessarily indicative of the presence of a cofactor other than TPQ. A recombinant methylamine oxidase from *Hansenula polymorpha*, which has been shown to contain TPQ, underwent an incomplete red-shift in 2.8 M KOH following derivatization with *p*-NPH (*16*). This phenomenon is more common with PH derivatives of TPQ enzymes (*23*), and it is likely that the nature of the protein pocket in which the cofactor is situated can influence the spectroscopic behavior of derivatized TPQ. This, in turn, might provide a reason, if not an explanation, for the bleaching effect of KOH on bovine SSAO−phenylhydrazone (*24*) and for the minor differences in λ_{max} values seen between TPQ enzymes from different sources.

Further evidence favoring the presence of TPQ and arguing against a C-2 linkage is seen in resonance Raman spectra of SSAO enzymes. While it is perhaps surprising that Raman spectra for derivatized TPQ enzymes are not more different from those for derivatized LO and the *A. niger* amine oxidase containing a Glu-linked TPQ, there nevertheless exist several distinguishing spectral features that suggest that the cofactor in SSAO is identical to that in other CuAO enzymes. Recently, the crystal structure of the *Escherichia coli* amine oxidase active site, which contains TPQ, has been determined to a resolution of 2.0 Å (*66*). Several features that might be expected to influence visible and resonance Raman spectra of the cofactor adduct were clearly demonstrated, including a putative active site base (Asp383), a tyrosine residue which hydrogen-bonds to O-4 of TPQ (Tyr369), and three copper

ligands (His524, His526, and His689). While all of the above features were conserved in the amino acid sequence for human placental SSAO (*60*), the His-X-His motif, which is conserved throughout all TPQ amine oxidases, was not present in the Glu-linked TPQ bacterial enzyme (*35*). These observations suggest that more substantial differences in active site structure might be present in a C-2-linked TPQ enzyme than were seen in the only fully sequenced placental SSAO. Unfortunately, the partial amino acid sequence obtained for rat adipocyte SSAO (*58*) is too short to contain any of the residues that constitute the active site.

The crystal structure of the *E. coli* active site also indicates that the ring of Tyr468, which is conserved in all known mammalian and bacterial amine oxidases, is inserted between the rings of TPQ and His526 (*66*). It is probable that this tyrosine residue has some mechanistic role, since its replacement with Phe in a recombinant bacterial histamine oxidase had a profound effect on enzyme activity and substrate specificity (*67*). Similarly, the Asp (or Glu in several bacterial CuAO enzymes) residue immediately following TPQ appears to be important in maintaining active site structure and cofactor position during catalysis, through an interaction with a fourth conserved His residue (*68*). Bovine and porcine plasma amine oxidases and the placental SSAO enzyme contain a valine immediately following TPQ-Asp-Tyr (*13, 60, 69*), and bacterial enzymes contain smaller residues such as glycine (*70*) in the corresponding position. In contrast, bovine and porcine SSAO enzymes have been shown here to contain a bulky tyrosine residue. It would be interesting to determine the effects on bovine plasma amine oxidase activity, substrate specificity, and particularly stereochemistry versus substrate tyramine of replacing Val473 (*8*) with a Tyr residue, so that the active site more resembles that of porcine aorta SSAO. The reason for opposite stereospecificities shown toward tyramine by plasma and tissue-bound amine oxidases in both cattle and pigs is unknown. With the assumption that the active sites of these enzymes bear some similarity toward that of the crystallized *E. coli* amine oxidase, any distortion of the position of Asp 471, or of Tyr472 with respect to TPQ470 and His521, might influence the substrate specificity and, perhaps, stereochemistry toward tyramine. Such an observation has been made in studies of a yeast alcohol dehydrogenase (*32*), in which replacement of an active site leucine with alanine resulted in reduced steric hindrance of cofactor rotation, with a subsequent 10 000-fold loss of stereospecificity toward substrate ethanol, but with virtually no loss of activity.

The cloned, human placental SSAO enzyme (*60*), which contains valine immediately following the TPQ consensus sequence, may thus possess an active site more similar in shape to bovine plasma amine oxidase (*8*), with which it shares 81% amino acid homology, than to porcine aorta SSAO. The stereochemistry of placental SSAO toward tyramine is not known. However, no two enzymes are known that have opposite stereospecificities toward a common substrate when their homology is greater than 50% (*32*). In view of the high degree of homology (83%) seen between human placental SSAO and rat adipocyte SSAO (*58*), and the many physical, physiological, and pharmacological similarities that exist between mammalian smooth muscle and adipocyte SSAO enzymes (*19–21, 65, 71*), plasma amine oxidases and tissue-bound amine oxidases may represent a unique group of closely related enzymes that are highly homologous but that have opposite stereospecificities toward a common substrate.

Although identification of a TPQ consensus sequence, along with confirmation of appropriate visible and Raman absorbance characteristics, is now considered sufficient proof of the presence of a TPQ cofactor (for example, *72*), further confirmation of the SSAO active site structure was sought by mass spectrometry. Peptide−(*p*-nitrophenyl)hydrazones purified from pronase and thermolytic digests of bovine and porcine SSAO, as well as PKDAO and PSAO as standards, were subjected to MALDI-TOF, laser-desorption (without matrix), positive and negative electrospray, fast atom (Xe) bombardment, and liquid secondary ionization (Cs^+) mass spectrometry. Experiments were done with samples purified in three different HPLC buffer systems, including TFA/acetonitrile, in several different mass spectrometers, and under a wide variety of experimental conditions chosen to optimize any parent ion signal detected. However, no identifiable parent ions were obtained from any of the samples, although a number of other peaks, which may have corresponded to fragments but which could not be related to a parent ion, were usually present. It has been reported that *p*-NPH derivatives of TPQ are prone to poor ionization and thus could prove impossible to detect by mass spectrometry (*36*). We were also unable to obtain a parent ion for peptide-phenylhydrazone purified from a thermolytic digest of PSAO (*13*). Since the *p*-nitro group withdraws electrons, thereby stabilizing the adduct, derivatization with hydrazines which contain an electron-donating group may facilitate ionization. For example, derivatization of ovine plasma amine oxidase with (*p*-methoxyphenyl)hydrazine (*p*-MeOPH) gave an adduct with $\lambda_{max} = 464$ nm ($\epsilon_{464} = 29\,000$ M^{-1} cm^{-1}), which shifted to 485 nm in 2 M KOH (A. Holt, unpublished observation). This compares favorably with the *p*-NPH derivative ($\epsilon_{457} = 36\,000$ M^{-1} cm^{-1}, shifting to 580 nm in 2 M KOH), and *p*-MeOPH may thus be useful as a chromophoric tag in future studies. Interestingly, while derivatization of a TPQ enzyme with PH takes from seconds to minutes to reach completion, and rather longer with *p*-NPH, formation of the TPQ−(*p*-methoxyphenyl)hydrazone was almost instantaneous.

Since the physiological substrate(s) of these SSAO enzymes is/are unknown, then stereochemical differences with respect to substrate tyramine may be physiologically unimportant. It is possible that a common endogenous substrate might be metabolized with identical stereochemistry by both plasma and tissue-bound enzymes. However, the finding that the active sites of porcine plasma and aortic SSAO enzymes are shaped differently due, at least in part, to the substitution of Tyr for Val in the sequence Asn-TPQ-Asp-Tyr-Val/Tyr, suggests that these enzymes metabolize different endogenous substrates. The N-terminal anchor sequences present in bovine and porcine enzymes, as well as results from experiments with nonpermeating inhibitors (*73*), suggest that the aortic enzyme is a Type II glycoprotein anchored to the plasmalemmae of smooth muscle cells and adipocytes. It would thus be ideally positioned to terminate the action of a neurotransmitter or of a paracrine mediator or neuromodulator released either from endothelial cells or from the adrenergic nerve plexus at the junction of the media and adventitia, in a manner analogous to that of acetylcholinester-

ase. Alternatively, SSAO produces hydrogen peroxide in high localized concentrations at the surface of smooth muscle or endothelial cells, or adipocytes, perhaps altering the reactivity of cell surface receptors (*74*), as well as contributing to oxidative damage of cells and tissues. Finally, it may protect vascular smooth muscle cells from the effects of circulating vasoactive or toxic amines. Since it seems that the plasma enzymes are ideally suited to serve this protective function (*75*), then a more site-specific role might be played by the tissue-bound enzymes. Increased knowledge of the cofactor and active site characteristics should facilitate the design of highly selective inhibitors (*76*) that might then be used in studies to identify the endogenous substrate(s) and function(s) of SSAO enzymes. It has been estimated that 14 million SSAO molecules are present on the surface of one adipocyte (*58*), making SSAO a predominant cell surface protein. Synthesis of this extremely large (160−180 kDa) and heavily glycosylated protein incurs a significant biosynthetic cost to the cell, and it is thus most unlikely that these enzymes are vestigial in nature. The potential involvement of SSAO enzymes in control of vascular tone and reactivity (*77*), fat cell metabolism (*78*), and cell growth and differentiation (*75, 79*) could provide a novel point of attack in the fight against ailments such as cardiovascular diseases, diabetes, and cancer.

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